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Chromosomal and nuclear distribution of the HindIII 1.9-kb human DNA repeat segment

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Abstract. A human interspersed repetitive DNA cloned in pBR322, the HindIII 1.9-kb (kilobase pair) sequence, was labeled with biotinylated dUTP and hybridized to acid-fixed chromosomes and paraformaldehyde-fixed whole cells in situ. Using our most sensitive detection techniques this probe highlighted on the order of 200 discrete loci, in punctate or banded arrays, that resembled a Giemsa-dark band pattern on chromosome arms. Interphase cells also displayed many discrete punctate spots of hybridization along chromosome fibers. The ubiquitous Alu sequence repeat also appeared to be concentrated in specific regions of the chromosome and predominantly highlighted Giemsa-light bands. Centromeric or ribosomal spacer DNA repeats used as controls in all studies gave the expected hybridization profiles and showed no non-specific labeling of chromosome arms. Cohesive groups of centromeric DNA arrays and rDNA clusters were observed in interphase nuclei. Refinements in methods for detecting biotin-labeled probes in situ were developed during these studies and calculations indicated that about 20 kb or more of the 1.9-kb repeat were present at each hybridization site. The chromosomal distribution of the 1.9-kb repeat suggests that this sequence may reflect, or participate in defining, ordered structural domains along the chromosome.

Introduction

Several families of interspersed repeat sequences have been found in high copy number in mammalian DNA (reviewed by Singer 1982). Two notable examples are (1) the short, and "ubiquitous" 300-bp (base pair) Alu sequences (Rubin et al. 1980; Jelinek et al. 1980; Pan et al. 1981) and (2) the longer 1.9-kb HindIII interspersed repeat (Maio et al. 1981; Schmeckpeper et al. 1981; Manuelidis 1982a, b). Most of the latter human DNA sequence can be visualized as a single 1.9-kb (kilobase pair) band on a gel after digestion with the restriction enzyme HindIII, and this band represents about 0.3% of the genome or about 4,500 copies per haploid cell (Manuelidis and Biro 1982). The observation that the 1.9-kb sequence can also be detected in longer restriction fragments, such as those visualized after cleavage with KpnI and other enzymes, is consistent with the proposal that the HindIII sequence defines a conserved region within a larger superfamily of reiterated sequences in primates (Singer 1982). The KpnI repeat is highly polymorphic and generates a series of fragments of variable

lengths when analyzed by Southern blotting (Adams et al. 1980; Maio et al. 1981; Manuelidis and Biro 1982; Kole et al. 1983). In addition, multiple internal rearrangements of blocks of sequence within this superfamily appear commonplace and this interpretation has been born out in a number of sequenced specimens (Thayer and Singer 1983; DiGiovanni et al. 1983; Potter 1984). Sequences homologous to regions of the Kpn superfamily have been found in roughly equivalent copy numbers in rodents (Manuelidis 1980, 1982b; Manuelidis and Biro 1982; Singer et al. 1983), as well as in primates (Maio et al. 1981; Singer 1982).

The conservation of these sequences suggests that they may be of some value to the organism. However, neither the reason for their existence nor the functions they may perform are presently understood. The Hind 1.9-kb sequences are not tandemly repeated and are therefore not likely to be found in a large "en bloc" arrangement on chromosomes, as are satellite DNAs. It is generally assumed that such interspersed repeats are ubiquitous and distributed throughout the entire genome fairly uniformly. This impression of dispersion is further supported by the detection of the 1.9-kb sequence in a large proportion of recombinant colonies (Shafit-Zagardo et al. 1982), as well as the detection of only a single copy of this repeat in most cloned fragments. Despite this, it is possible that such sequences are distributed on chromosome arms in a specific, nonrandom fashion and thus could subserve some chromosome indexing or recognition functions (Manuelidis 1982a, Manuelidis and Biro 1982). An orderly arrangement of such sequences in chromosomal DNA would not be readily apparent in cloning and restriction enzyme experiments where the genome is digested into relatively short linear fragments.

To study the question of chromosomal distribution in more detail a series of in situ hybridization experiments were done using the 1.9-kb HindIII fragment as a probe. It was necessary to develop high resolution methods for in situ detection of DNA fragments with multiple chromosomal loci, since preliminary studies showed that autoradiographic detection of such fragments was insufficiently precise for unambiguous assignment to specific chromosome domains. In contrast, the use of biotin-labeled DNA probes, coupled with immunological detection techniques, appeared to offer a promising approach to achieve the resolution required (Manuelidis 1982a). We have refined the detection methods for visualizing biotinylated probes and have detected a reproducible pattern of hybridization for the HindIII 1.9-kb interspersed repeat. We show here that

this repeat sequence appears in nonrandom clusters on the arms of all chromosomes in a pattern very similar to the dark bands of Giemsa banding. Such an arrangement raises the possibility that certain repeated DNA subsets may participate in defining ordered domains along the chromosome.

Materials and methods

Cloned DNAs. The 1.9-kb HindIII interspersed repeat in pBR322 was isolated as described (Manuelidis and Biro 1982). A cloned 340-bp centromere repeat was a generous gift of J. Maio and was similar in sequence to the consensus 340-bp sequence (Wu and Manuelidis 1980; J. Maio, personal communication). A Bam HI variant of the 340-bp sequence with a high affinity for the X chromosome was a gift of H. Willard (Willard et al. 1983; Yang et al. 1982). A nontranscribed spacer fragment adjacent to and contiguous with the human 18S ribosomal gene was isolated and subcloned in pBR322 (Manuelidis and Biro, unpublished); the 3' end of this 6-kb repeat maps to about 200 bp 5' to the end of the 18S gene sequence (Chen and Manuelidis, in preparation). One or more of these sequences were used as controls in all hybridization experiments. Several interspersed repeats such as the Alu sequence (gift of C. Duncan and S.M. Weissman; Pan et al. 1981) were also used as controls in some experiments.

Purified DNAs were nick translated using biotinylated derivatives of dUTP as described (Manuelidis et al. 1982; Brigati et al. 1983) using DNase I concentrations that yielded fragments ranging in size from 50–500 bp. DNAs were labeled with either Bio-4-dUTP, Bio-11-dUTP, or Bio-16-dUTP derivatives, which differ in the number of atoms (4, 11, or 16) present in the linker arm that connects the biotin moiety to the 5 position of the pyrimidine ring (Brigati et al. 1983).

Chromosome hybridization. Normal human metaphase chromosomes were prepared from whole blood cell cultures, stimulated with phytohemagglutinin, and partially synchronized with methotrexate using standard protocols. Chromosome spreads were incubated at 37° C for 1 h in 100 µg/ml RNase in 2 × SSC (sodium chloride/sodium citrate) – 2 × Denhardt's buffer (Denhardt 1966), dehydrated in ethanol, and dried with an air jet. They were then (most reproducibly) denatured at 70° C for 2 min in 70% formamide-2 × SSC as described by Harper and Saunders (1981), dehydrated, and dried. Preparations with overly pale chromosomes or chromosomes showing halos in phase contrast were useless for localization of interspersed repeated DNAs. Biotinylated probes were denatured at 95° C for 4 min in a small volume of water, quenched on ice, and the remaining components of the hybridization mixture added. The final hybridization mixture contained 10% dextran sulfate, 40% formamide (distilled), 4 × SSC, 1 × Denhardt's buffer, 10 mM sodium phosphate, pH 6.8, 70 ng/ml of biotinylated probe DNA, and 250 µg/ml of sonicated salmon sperm (carrier) DNA. If dextran sulfate was omitted, the probe was denatured and applied in 50% formamide-2 × SSC. The hybridization mix was immediately added to the dry slides, covered with an acid-washed coverslip, and sealed with rubber cement. Highest hybridization sensitivities were obtained using 10% dextran sulfate in the hybridization mixture, but with this reagent it was essential

to use probe concentrations of <100 ng/ml and to follow with rigorous posthybridization washings. Slides were hybridized at various temperatures (30°, 34°, 39° C) in a moist chamber overnight. They were then washed extensively in 2 × SSC, followed by a wash in 2 × SSC-5 mM ethylenediaminetetraacetate (EDTA), pH 7.4, at 60° C for 15 min and allowed to cool to room temperature. Slides were then washed in 0.1 × SSC at 45° C for 30 min, equilibrated in phosphate-buffered saline (PBS), blocked with 10% serum for 10–15 min and treated at 37° for 1–2 h with Avidin-peroxidase conjugates (ABC reagent, Vector Laboratories). A three-step detection method, employing rabbit anti-biotin antibody, biotinylated goat anti-rabbit IgG and the ABC complex was required to detect equivalent hybridization signals when the probes were hybridized in 50% formamide-2 × SSC without dextran sulfate (Manuelidis et al. 1982). After extensive washing in PBS, slides were developed for 5–10 min with diaminobenzidine (DAB)-H₂O₂, and in selected cases preparations for electron microscopy were made as described (Manuelidis et al. 1982).

The following comments summarize observations made during the more than 50 hybridization experiments done to delineate optimal resolution and detection protocols. Extensive repeated acid-methanol fixation, including an overnight fixation at 4° C, were necessary for optimal chromosome preservation. Postfixation with 4% paraformaldehyde before or after denaturation did not improve signal intensity or resolution, while 0.5% glutaraldehyde fixation decreased signal strength. The most stable chromosome spreads for denaturation and hybridization were made on acid-cleaned slides. The use of various subbing solutions in most cases increased the nonspecific background of peroxidase reaction products, especially in experiments where dextran sulfate was added to the hybridization mix. Blocking slides with triethanolamine gave variable results and was considerably less important than the denaturation and hybridization conditions (vide infra). Blocking of nonspecific adherence sites on slides was more conveniently done by including Denhardt's buffer in the RNase incubation step. Spreads made on plastic coverslips displayed very little nonspecific background, but dispersion of chromosomes was less reproducible on plastic. Mike Watson and Valerie Lindgren kindly provided some of the chromosomes used in these studies.

Tissue culture cells. Human glioma cell lines TC620 and TC593 used in this study have been previously characterized (Manuelidis and Manuelidis 1979). For in situ hybridization optimal morphological preservation and probe penetration were obtained by lightly fixing cells on coverslips with 4% paraformaldehyde in PBS for 5 min and then immediately incubating them with 0.5% Triton X-100 in PBS (2 × 10 min). Electron microscopical analysis showed that most of the plasma membranes were removed by this step (data not shown) yet the nuclei appeared to remain intact during subsequent procedures or storage in PBS (up to 4 weeks). The Triton extraction step is usually sufficient for both probe and antibody penetration of the nucleus. Additionally, freeze-thawing in liquid nitrogen (three times) of fixed cells, equilibrated in 20% glycerol in water, was also used to facilitate probe penetration (Manuelidis 1984a) and to avoid pronase treatment (Brigati et al. 1983). To preserve three-dimensional structure, cells were never dried, but were equilibrated in hybridization buffer (50% forma-

mid-2 \times SSC) without dextran sulfate, and then denatured together with probe DNA. Sealed coverslips were rapidly brought to 78°C for 2 min in a closed humidity chamber as described (Brigati et al. 1983). The chamber was opened and drained to decrease the temperature, reclosed, and incubated at 35°C overnight. Coverslips were washed in 50% formamide-2 \times SSC, and in 0.05% Triton X-100 in PBS to decrease nonspecific background. Subsequent detection steps with antibody were as above.

Several experiments were also done with 0.5- to 1.0- μ m cell sections. Paraformaldehyde-fixed cell pellets were washed extensively in PBS, dehydrated, and embedded in methacrylate for hybridization as suggested by J. Gall (personal communication). For proper polymerization it was necessary to absorb impurities present in commercial methacrylate (Polysciences) by stirring the methacrylate stock solutions with activated charcoal for 1 h. The stock solutions were filtered and stored at 4°C. Tissue was conventionally infiltrated from ethanol into a one-to-one ratio with a 7:3 mixture of butyl:methyl methacrylate, and then equilibrated in methacrylate mixed with 1% benzoyl peroxide. Methacrylate polymerization was carried out at 60°C overnight in tightly capped polypropylene Eppendorf tubes, since polyethylene Beem capsules allowed too much air penetration and resulted in poor polymerization. Sections of 0.8 μ m were cut and then dried at 60°C on slides that had been subbed with 0.5% Elmers glue in water. The methacrylate embedding media was then removed from the cells by two 10-min changes in xylene (per J. Gall). The cells were rehydrated (without drying) to PBS, and equilibrated with hybridization mix (containing dextran sulfate) for 15 min. Hybridization mix with probe DNA was then applied, the coverslips sealed, and the preparations denatured in a humidity chamber as described above. Posthybridization washes and peroxidase detection of hybrids were as done for chromosomes. Cells were also embedded in diethylene glycol distearate (DGG) as described (Capco et al. 1984) and sections free of embedding media similarly hybridized. The morphological preservation and handling properties were better with methacrylate, but more intense and evenly distributed labeling was seen with DGG-embedded sections.

Signal intensification. A number of hybridizing loci on chromosome arms were very faint or almost invisible (especially in photographic reproduction). Phase contrast increased the optical density of peroxidase products, but individual labeled sites that were closely juxtaposed were less resolved with phase optics. We found that gold and silver intensification (Gallyas et al. 1982; Newman et al. 1983a) of DAB was quite helpful in delineating these minor sites, especially when it was combined with enhanced video contrast and Nomarski optics (see Fig. 4A) set up as described (Inoué 1981; Manuelidis 1984a). The intensification procedure used (Newman et al. 1983a) was more sensitive than a second simplified procedure (Newman et al. 1983b) or than OsO_4 treatment; the latter also increased the density of unlabeled structures. Slides or coverslips were rinsed five times in water and treated with 0.1% gold chloride for 5 min, rinsed five times in water and covered with 0.17% neutralized sodium sulfide for 5 min (0.5 ml 3.5% sodium sulfide stock solution was added to 9.5 ml of 100 mM of Tris/acetate, pH 7.4). Slides were then rinsed five times in water and agitated for 50–60 s in freshly mixed physical

developer (made as described by Gallyas et al. (1982) using baked tungstosilicic acid). The reaction was stopped by a 30- to 60-s rinse in 1% acetic acid, followed by several rinses in water. Control studies showed that at least 10 μ m of tissue was adequately penetrated with these incubation times; more than 70 s in physical developer brought out nonspecific background, especially in slides with residual bumps of dextran sulfate. The reaction worked equally well on acid-fixed or paraformaldehyde-fixed preparations with equivalent incubation times. To test if this intensification procedure was increasing the sensitivity of detection or only enhancing the optical density of the signal, dilutions of peroxidase were dotted onto nitrocellulose paper, dried, blocked with Denhardt's buffer, and intensified as above. There was only an equivocal increase in peroxidase detection sensitivity with intensification (possibly two-fold) in contrast to the report of Gallyas et al. (1982). The density of the reaction product was considerably enhanced, and if photographed would appear as a ten-fold or greater increase in detection sensitivity. This density enhancement was essential for the visualization of more minor hybridization sites by light microscopy, and was also quite useful for high resolution electron microscopic detection of labeled DNA sequences (Manuelidis, unpublished).

Several experiments were also done using avidin-biotinylated alkaline phosphatase complexes (Leary et al. 1983). With extended development times (> 30 min without levamisole) significant endogenous background was obtained. In addition, the label was not as well defined or discrete as that obtained with peroxidase development.

Results

In autoradiographic experiments clusters of grains were apparent on some regions of chromosome arms following hybridization with a ^3H -labeled HindIII DNA repeat probe (Manuelidis 1982a), but with longer exposures to obtain more sensitivity these regions of labeling were not sufficiently resolved from each other to permit an unambiguous analysis. Thus it was not possible to distinguish a diffuse distribution of this sequence from a clustered or focal distribution. We therefore sought to improve the resolution using biotin-labeled probes followed by immunological detection methods. Initial studies were done without dextran sulfate, and hybridized, biotin-labeled DNA was detected with anti-biotin antibodies followed by fluorescein-tagged second antibodies. These studies showed fluorescence confined to the chromosomes, with little scatter of signal. However, the results were not clear cut, as the fluorescence signal was weak, and without computerized data collection with an intensifying camera, as recently described for detection of single-copy genes with biotinylated probes (Albertson 1984), it was impossible to distinguish differences in intensity of label along the chromosome arms. In contrast, control hybridizations done at the same time using ribosomal spacer DNA probes gave a bright fluorescence signal confined to the tips of all acrocentric chromosomes (data not shown).

Use of peroxidase-tagged reagents for detection of hybridized probe yielded a crisper and more unambiguous localization of label. Hybridization mixes (without dextran sulfate) that contained probe DNA labeled with Bio-4-dUTP or Bio-11-dUTP yielded a banded pattern on some metaphase spreads, but there was variability in labeling

from cell to cell. Some chromosome spreads showed relatively few bands, or a punctate label, that were confined to a few discrete regions over the chromosome arms. This type of labeling pattern can be seen in the electron micrograph shown in Figure 1A. Many of these chromosomes displayed symmetrical punctate label on each sister chromatid, suggesting that these were bona fide loci of the 1.9-kb sequence. Some labeled sites, however, were not represented equally on each sister chromatid. This observation most likely reflects the relative inefficiency of the *in situ* hybridization process when probing for target sequences at low abundance in a single locus. Indeed, previous studies using ^3H and ^{125}I -labeled probes to map single copy genes (Harper and Saunders 1981; Gerhard et al. 1981) had shown that only 60%–70% of the spreads analyzed showed hybridization to one chromosome in the diploid set while only 20%–30% of the metaphases showed hybridization to both target chromosomes in the karyotype. The fact that only 25%–30% of the hybridization foci in the chromosome spread in Figure 1A were present on both chromatids (see arrows) is thus not surprising, and well within statistical

expectations. In contrast, control slides hybridized with a localized, high-copy-number probe, the rDNA spacer sequence, consistently showed a definitive labeling pattern that was confined to virtually all the appropriate acrocentric sites without any labeling of the chromosome arms (Fig. 1B). It is of note that this ribosomal DNA spacer, which has not been previously used for *in situ* hybridization, gave the same pattern of hybridization signal as that observed previously with probes specific for the 18S and 28S genes themselves (e.g., Evans et al. 1966).

The above experimental results with the 1.9-kb probe also suggested that we were detecting major sites of this repeat, but that other sites were only marginally detectable. The representation of the rDNA sequences at each acrocentric site can be estimated to be about 500 kb (6 kb rDNA spacer \times 400 copies of rDNA cistrons/5 acrocentric chromosomes). The rDNA signal was always considerably more intense than the 1.9-kb HindIII signal in identically processed slides. From both numerical calculations and relative signal intensity, it was apparent that we were detecting only part of the HindIII 1.9-kb repeat with these methods, possi-

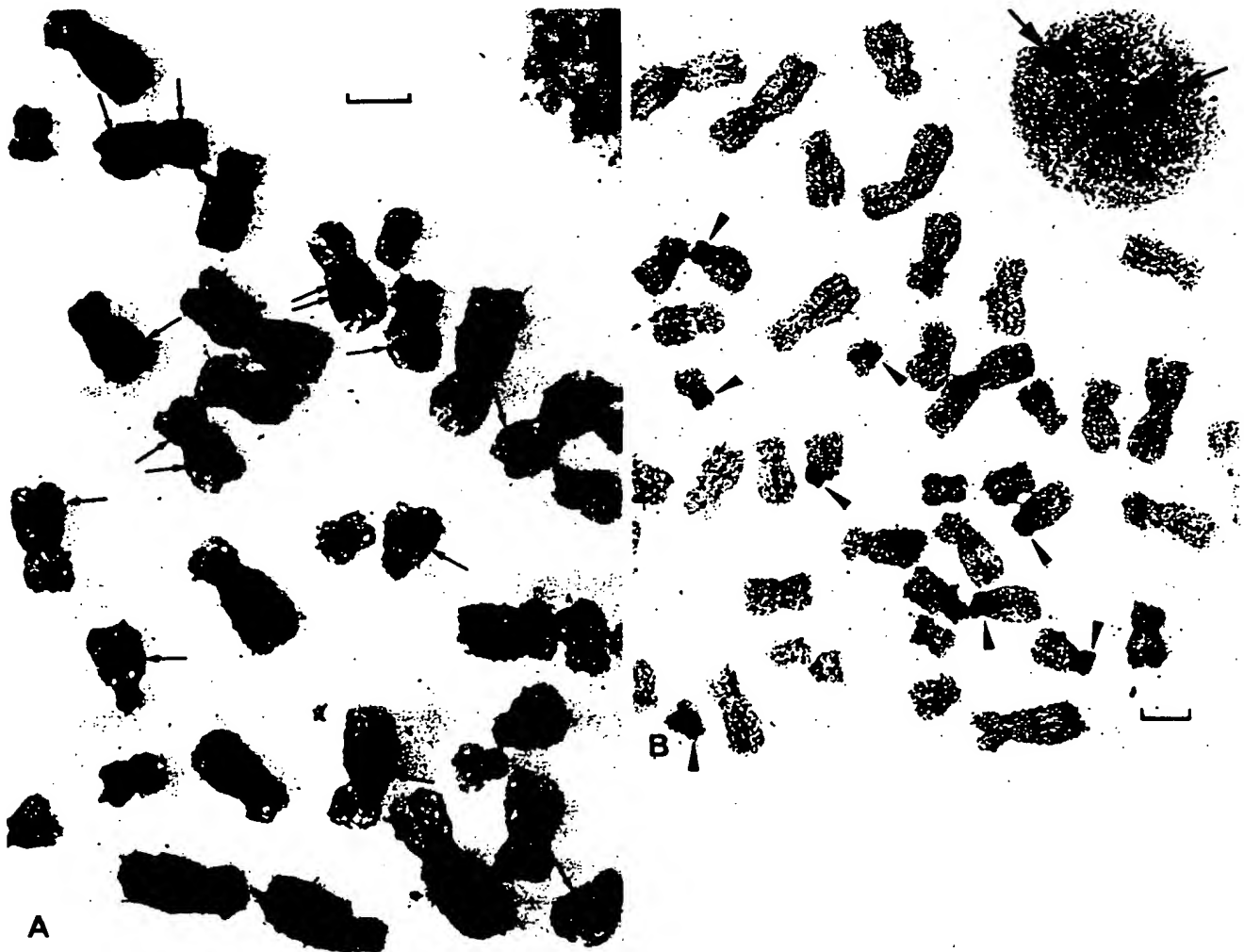


Fig. 1. The 1.9-kb HindIII repeat (A) and ribosomal spacer DNA (B) were labeled with Bio-4-UTP and hybridized to human chromosomes at 60°C in $2 \times \text{SSC}$ (without dextran sulfate). Hybridizing sites were detected using rabbit anti-rabbit IgG tagged with peroxidase, and chromosomes were prepared for electron microscopy as described (Manuelidis et al. 1982). Note small punctate regions of label on most chromosome arms in A. Many of these labeled regions are detected on both sister chromatids at the same locus (arrows). No label is seen over chromosome arms in the rDNA hybrid (B), but acrocentric regions show dense reaction product (arrowheads) and the nucleus shows dense nucleolar label (arrows). Bars represent 2 μm .

bly only a subset of family members that were clustered together in high numbers at certain loci on chromosome arms.

We therefore attempted to increase our detection levels while maintaining the resolution necessary to distinguish closely spaced bands. Ideally it would be advantageous to be able to localize sites of hybridization without resorting to extensive statistical counting or scoring for identification of labeled bands, although this would be difficult when analyzing the distribution of a highly repetitive sequence. Hybridization of chromosomes with probes containing Bio-16-dUTP, combined with dextran sulfate in the hybridization mixture, resulted in higher labeling intensities on chromosome arms, as well as more reproducible patterns of labeling. Many bands could thus be resolved on all chromosome arms with prometaphase preparations showing the highest number of distinct bands.

Several experimental parameters were important in achieving reproducible hybridization results. Banding (and signal) were lost if the chromosomes arms were overdenatured, probably due to loss of DNA from chromosomes; the centromere region was less critically affected by overdenaturation. ^3H -Labeled chromosomes studied with autoradiography confirmed this interpretation (data not shown). It was also extremely important to extensively wash the preparations to remove the dextran sulfate in these more sensitive hybridization protocols. This was especially true if electron microscopy was used for analysis, since any extra material would be visualized as an electron-dense focus, regardless of whether it contained peroxidase products.

In a typical experiment with dextran sulfate and Bio-16-dUTP labeled probe, strongly hybridizing bands were seen without resorting to intensification procedures. Avidin-peroxidase conjugates could also be used directly without an intermediate antibody step. Additional bands were brought out after initial viewing using the heavy metal intensifica-

tion procedure. Representative spreads after intensification are illustrated in Figures 2 and 3, which show hybridization signals obtained at different stringencies. Although not every chromosome in each spread gave identical hybridization profiles, most spreads showed characteristic and identifiable banding patterns, especially of chromosomes 1, 2, 4-7, 9, 11, 20, and 21. However, to establish that a reproducible pattern of hybridization was obtained with the HindIII probe it was necessary to analyze and compare numerous chromosome spreads. This banding pattern closely resembled a Giemsa-dark banding pattern. There was little diffusion of signal, and label was essentially confined to the chromosome, i.e., the resolution was high. The bands were not as perfect as the bands of good G-banded chromosomes since there were some unavoidable distortions due to denaturation (see Discussion). Nomarski optics combined with enhanced video contrast clearly resolved many minor bands that were difficult to reproduce directly with photography (Fig. 4A). Occasionally an anomalous banding pattern was seen in a single chromosome of a spread (e.g., chromosome "3", Fig. 3) but this was observed infrequently and was most likely a distortion artefact due to denaturation. The lack of labeling at centromeric regions of chromosomes 1, 9, and 11 was also clear cut even when the combined protocol of dextran sulfate hybridization, antibody detection, and heavy metal intensification (the most sensitive procedure thus far) was employed. However, with this latter method, the more heavily labeled bands on chromosome arms could not be easily resolved from each other. In control slides hybridized with centromeric repeats, this high sensitivity detection protocol still yielded label confined only to the centromere region, with no detectable label on chromosome arms (e.g., Fig. 4B).

Changing the stringency for reassociation, by hybridizing at temperatures ranging from 30°-40° C in formamide, did not appreciably alter the 1.9-kb labeling pattern. More sen-

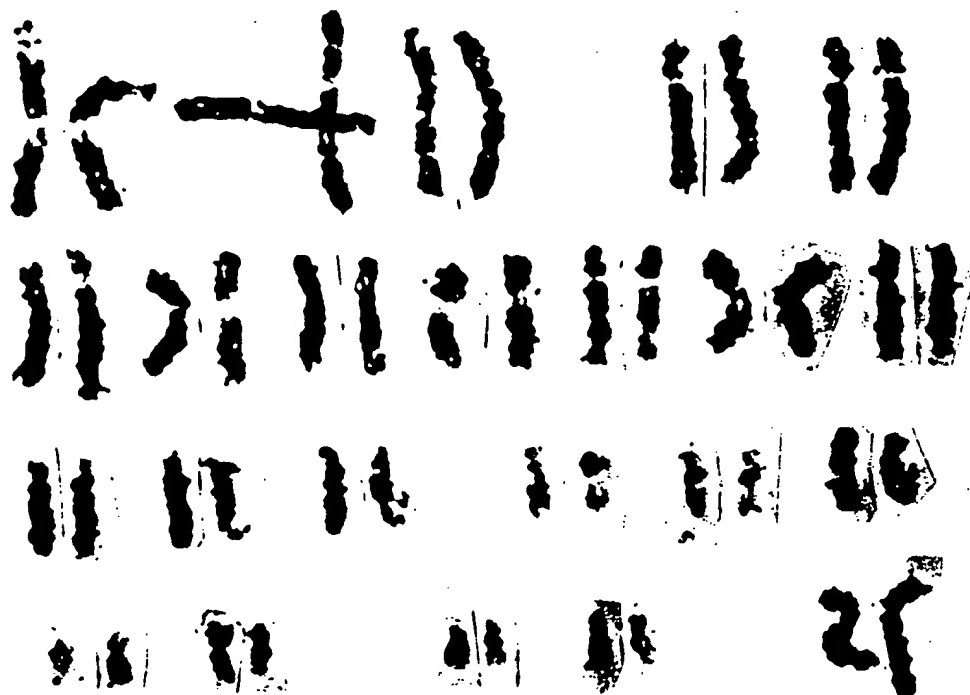


Fig. 2. Typical single-cell spread of chromosomes hybridized with the 1.9-kb repeat at 34° C in formamide with dextran sulfate. Probe (labeled with Bio-16-dUTP) was detected with avidin-biotinylated peroxidase complex without antibody, and the signal intensified with heavy metal ions. Chromosomes were photographed under phase contrast to bring out minor sites of hybridization. Chromosomes 1, 2, 4, 6, 7, 11, 20 and 21 show characteristic dense bands that correspond to a Giemsa-dark banding pattern



Fig. 3. Spread of chromosomes treated as described in Figure 2 except hybridization was under higher stringency conditions (at 39° C). Certain chromosomes are easily identified by their Giemsa-like banding e.g., 1, 2, 4-6, 9 and 20. One chromosome "3" shows an anomalous or variant banding pattern that was not reproduced in other spreads

sitive detection procedures, i.e., heavy metal intensification or the use of antibody steps, were necessary to bring out the same bands at the higher temperature, although many bands were still detected at 39° C without intensification. The latter temperature represents a reasonably high stringency condition for biotin-labeled probes, since their T_m (the mean melting temperature of the DNA helix) is lower than that of biotin-free probes (Langer et al. 1981). In similar stringency experiments, control slides were hybridized to an X-centromere specific repeat that is partially homologous to the 340-bp Eco RI centromeric repeat. This clone showed strong hybridization to the X-centromere and a few other centromeres at 39° C (data not shown), but when these preparations were intensified, almost all centromeres were labeled (Fig. 4B). Autosomal chromosomes are known to contain the Bam HI variant centromeric repeat in lower copy numbers than the X chromosome (Willard, personal communication). This control demonstrated our ability to unambiguously detect, without significant background, more minor sites of hybridization and reinforced our contention that the additional 1.9-kb HindIII sequence sites observed following heavy metal intensification were not artefactual.

To test the specificity of the banding pattern observed with the 1.9-kb repeat, another interspersed repeat sequence was also used in control studies. A cloned Alu sequence was nick-translated and hybridized under standard conditions. This probe sequence was more easily detected on chromosome arms than the 1.9-kb HindIII repeat and was concentrated at different chromosomal sites than the HindIII repeat. A comparison of the hybridization patterns on several chromosomes 1 are shown in Figure 5. Note that a telomeric region (1p3) was heavily labeled with the Alu

probe but not appreciably labeled with the HindIII probe. Thus the Alu hybridization highlighted a Giemsa-light or R-band region. In contrast, the Hind repeat appeared in general to be clustered in Giemsa-dark regions. Figure 5 also shows the reproducibility of these patterns in the chromosomes 1 of several cells. In all cases the Alu repeat, unlike the Hind repeat, was easily visualized without intensification as would be expected from relative copy numbers. The fact that we obtain no signal at all (on any chromosome) when single-copy gene probes (e.g., a 1.5-kb globin sequence) are hybridized provides further evidence that the 1.9-kb HindIII and 300-bp Alu sequences reside in discrete regions of the chromosomes.

From the above studies it was apparent that most of the HindIII 1.9-kb sequences were clustered together in a nonrandom fashion. Furthermore, the clusters were not equal in intensity. Some bands appeared to contain many copies of this repeat, whereas others contained fewer copies and thus were brought out only by intensification. Because the most sensitive detection procedures often lead to intense labeling in some loci, adjacent chromosome regions can often be obscured. Thus it was not possible to determine if a single or a limited number of the 1.9-kb sequences were present in the "unlabeled interband" regions.

At least 250 foci of hybridization were detected in the entire karyotype with the 1.9-kb HindIII probe. If all sites contained equal numbers of the 1.9-kb sequence, each band would represent on the average about 20 copies of the 1.9-kb repeat. These calculations indicate the hybridization procedures used to detect most of these sequences is about ten-fold more sensitive than the procedure to visualize the 500-kb clusters of ribosomal DNA. Since a variety of unpublished studies indicate that we require about 20 kb of

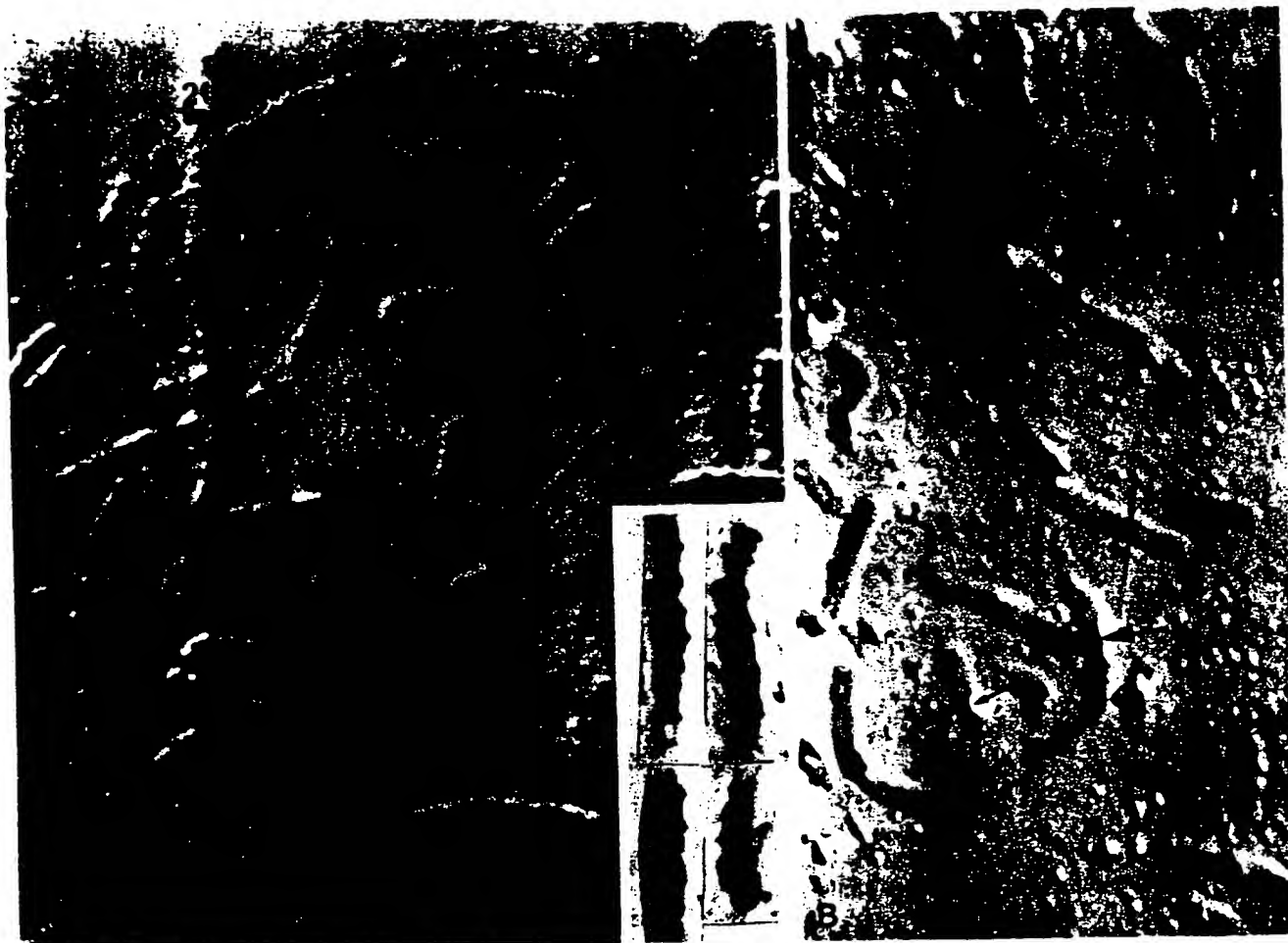


Fig. 4A. Low-power video display of Nomarski image of chromosomes hybridized at 39° C to the 1.9-kb repeat. Note that with these optics many more bands, or punctate regions, are resolved, e.g., on chromosomes 1 and 2. *Inset* shows higher-power magnification of a few of these chromosomes: *top row*, both chromosomes 6; *bottom row*, chromosomes 11 and 13. Bands are clearly visible, and the density of signal on the tip of 11q is less intense than bands in the center portion of 11q. Banding is comparable to Giemsa-banding. On the *right* is an identically processed spread probed with the X-centromere repeat. In addition to several heavily labeled centromeres (*large arrows*) a few minor sites on other centromeres are apparent (*small arrows*) but no label is seen on chromosome arms. "Bumps" in background using Nomarski optics are residual dextran sulfate clusters



Fig. 5. The four chromosomes 1 on the *left* have been hybridized with the Alu repeat probe and the signal is readily apparent without intensification. The five chromosomes 1 on the *right* were hybridized with the Hind repeat and are illustrated following heavy metal intensification. Note that the 1p3 region is densely stained with the Alu probe but is relatively unlabeled with the Hind repeat. Each hybridization pattern is clearly different. Alu is diffusely concentrated while the Hind repeat is more discretely clustered

target sequence at a single locus to generate a visible signal with the biotin-specific detection reagents employed, even with heavy atom intensification, we suggest that the minor sites of hybridization contain as few as ten copies of the 1.9-kb HindIII sequence. Although the level of hybridization at each site cannot be directly quantified as it can be by using autoradiographic detection methods, it is possible to obtain semiquantitative data by analyzing the color

intensity of the peroxidase products. A computer procedure for such analyses will be presented elsewhere (Manuelidis, in preparation).

To further assess the chromosomal distribution of the 1.9-kb HindIII sequence, the probe was hybridized to para-formaldehyde-fixed whole cells. Many punctate spots of hybridization were observed along interphase chromosome fibers (Fig. 6A). The tortuous course of labeled interphase



Fig. 6. Hybridization of paraformaldehyde-fixed whole cells to the 1.9-kb repeat (A) and the rDNA spacer (B). Hybridization was done at 35° C in 50% formamide – 4×SSC without dextran sulfate; probes were labeled with Bio-16-dUTP and were detected with antibodies as in Figure 1, without metal ion intensification. A Interphase chromosome fibers are labeled with the 1.9-kb repeat, and these fibers follow a somewhat tortuous course (e.g., at *arrows*). These chromosomes showed punctate dark spots along such chromosome fibers at higher magnification (no OsO_4 treatment). B Cells hybridized with the rDNA probe show no label over chromosome arms, except in the nucleolar region, even when treated with OsO_4 as shown here. Bars represent 3 µm

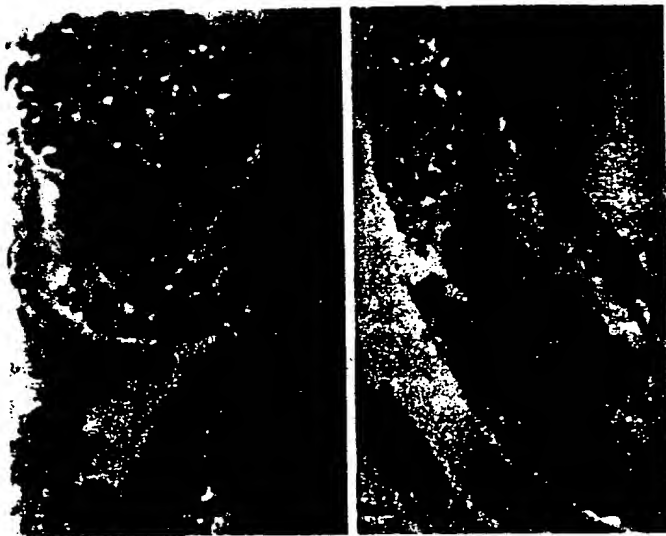


Fig. 7. Thin sections of whole cells hybridized to the 1.9-kb repeat (A) or to the X-centromere repeat after embedding in methacrylate and removal of the embedding media (B). Hybridization conditions and hybrid detection were as in Figure 2. In A, dense punctate regions are labeled and well resolved periodically along interphase chromosome fibers, especially in the giant nucleus at *top*. B In contrast, the X-centromere clone shows only a few large collections of label adjacent to the nuclear membrane with no labeling of other interphase chromosome fibers. Video display, phase contrast. Bars repeat 1 µm

chromosomes (0.2 µm wide) could be followed in optical-through-focus in these three-dimensionally preserved nuclei. No congregation or aggregation of labeled regions was apparent in these paraformaldehyde-fixed interphase nuclei. Thus, in contrast to centromeric repeats in interphase nuclei (Manuelidis 1984b), these 1.9-kb repeats did not form larger foci of nuclear organization. In other control studies, ribosomal DNA sequences, like centromeric repeats, were also collected together into a few organized or associated

arrays. Without intensification, hybridized ribosomal arrays were essentially identical to the active 0.2-µm-wide silver-stained nucleolus organizer region (NOR) arrays in the nucleolus (Manuelidis 1984a). Figure 6B shows the intense hybridization of ribosomal sequences to the nucleolus of whole cells. In this case the label is less well resolved (or overdeveloped) due to OsO_4 intensification, which was done purposely to show that in this control there was no detectable label over most chromosome regions occupied by the 1.9-kb HindIII sequence.

Since the pattern of hybridization of the 1.9-kb HindIII sequences was quite complex in whole cells, we attempted to study the hybridization pattern of this sequence in 0.8-µm sections of paraformaldehyde-fixed cells. Again, many punctate (about 0.2 µm) regions of hybridization were observed along interphase chromosome fibers (Fig. 7A). The X-centromere probe yielded large focal collections of centromere-specific arrays (Fig. 7B), in sharp contrast to the distribution of the 1.9-kb probe. The pattern of hybridizations observed in DGG-embedded sections were similar to those shown in Figure 6 with methacrylate-embedded material (data not shown). Collectively, these results thus demonstrate that the 1.9-kb HindIII sequence is clustered into numerous discrete foci both in the interphase nuclei and in mitotic chromosomes.

Discussion

The preceding results indicate that a significant portion of the HindIII 1.9-kb repeats are “clustered” together at discrete loci on chromosome arms and are not randomly distributed throughout the chromosome. Since this sequence is a conserved segment of the Kpn superfamily, the data also imply that most, if not all, of the larger repeat unit is also focal within the chromosome. It is known from hybridization and cloning studies (Adams et al. 1980; Manuelidis and Biro 1982; Shafit-Zagardo et al. 1982) that most of these sequences are not directly contiguous. Since we cannot as yet unambiguously detect a single 2-kb copy

of DNA using biotin-labeled probes (our detection limit is about 20 kb) the punctate or banded sites seen with the HindIII 1.9-kb probe are likely to represent multiple copies of this sequence in a highly defined, periodic arrangement that reflects, or is part of, a higher-order chromosome organization. For example, in either a looped (Marsden and Laemmli 1979), or in a helically wound (Sedat and Manuelidis 1978) model of chromosome folding, each repeat could be separated by 50–100 kb of other DNA and still lie adjacent to another family member, e.g., at the base of each loop or coil. Thus ten or more copies of this interspersed repeat would be localized at each discrete chromosomal site where hybridization is observed.

It is most intriguing that the pattern of labeling closely resembles a Giemsa-banding pattern. Although the molecular basis and meaning of Giemsa banding is not entirely understood, at the very least the differential trypsin sensitivity of bands suggest that special proteins may reside at similar and specific loci. More recently, DNA gene sequences that tend to be late replicating or transcriptionally inactive have been postulated to reside in Giemsa-dark bands, whereas active-tissue-specific genes and "housekeeping" genes have been associated with R-bands, or Giemsa-light regions that are early replicating (Holmquist et al. 1982; Goldman et al. 1984). Middle repetitive sequences fall into both categories. It is of interest that the 1.9-kb HindIII sequence tends to replicate late, although not as late as centromeric repeats (Holmquist and Manuelidis, unpublished) and the banding pattern is similar to that of a Giemsa-dark pattern. In contrast the Alu pattern (see Fig. 5) resembles a Giemsa-light or R-banding pattern that would be compatible with early replicating domains. Indeed, Alu-like sequences in human cells and Alu-like sequences in hamster cells have been found recently to be early replicating (Holmquist, personal communication). It is possible that the 1.9-kb HindIII sequence or members of the Kpn superfamily, together with a special group of proteins, could constitute structural arrays that would have some meaning in organized processes such as replication, segmental transcription, or site-specific recognition in Giemsa-dark regions. The conservation of these sequences in mammalian evolution (Maio et al. 1981; Manuelidis 1982a; Singer et al. 1983), as well as their ordered arrangements, as observed here, is compatible with a significant "functional" role for these sequences.

Since so many bands are present, one might reasonably ask how any specificity for individual bands may be obtained. First, the Kpn I repeats form an enormous pool of common elements, yet family members are not identical in sequence (Manuelidis and Biro 1982; Lerman et al. 1983). Second, either "scrambling" or orderly rearrangement of these sequences (Manuelidis and Biro 1982; Lerman et al. 1983; Weissman and Rogan, personal communication) and their sequence variants, as well as actual numerical representation at each clustered site, could confer specificity, and at the same time provide a common element for basic recognition in specific nuclear processes. Such an element would presumably be selected for in evolution and thus provide a reason for its maintenance in large numbers. While the focal distribution of the 1.9-kb HindIII sequence in both interphase and mitotic chromosomes suggests that repeat elements may participate in higher-order nuclear organization, some members may also have other roles as well. Singer (1982) and Martin et al. (1984) have reported

that some Kpn I family members contain long, open reading frames and thus may have been, or are (at least in part), protein coding elements. Although transcription of some Kpn I members can be interpreted as consistent with a protein coding function, transcripts homologous to the Hind 1.9-kb sequence are predominantly localized in the nucleus (Shafit-Zagardo et al. 1983; Lerman et al. 1983; Kole et al. 1983). A transcriptionally important product would also not explain the high-copy number of this repeated element in the genome; transcription might conceivably be associated with a subset of these sequence, i.e., those that may be more dispersed, or not clustered in periodic arrays. Although transcription in itself does not absolutely indicate coding related functions since even satellite DNA in some instances may be transcribed (Varley et al. 1980), some DNA sequences have been found to encode gene products and to provide specific recognition sites [e.g., the internal promoter sequences of RNA polymerase III transcribed genes (Sakonju et al. 1980; Bogenhagen et al. 1980)]. While it is also possible that the 1.9-kb HindIII sequence is, or was, part of a transposable element (Georgiev et al. 1982; Sharp 1983) that acquired its present chromosomal localities by specifically inserting into nonfunctional DNA, where it would be selectively harmless, it is equally feasible that its distribution in the chromosome reflects a position-dependent function. If both the HindIII and Alu sequences or their ancestors are, or were, mobile and inserted into nonfunctional DNA, it would be surprising that these two sets of sequences would insert into two distinct arrangements as observed, yet span virtually the entire chromosome complement. Similarly if the HindIII or Alu sequences were merely neutral hitchhikers in the genome (Doolittle and Sapienza 1980), one would not a priori expect to find an ordered and clustered arrangement of such sequences within the mitotic chromosome.

As noted before the Kpn superfamily is highly polymorphic not only with respect to single base changes (e.g., restriction sites) but also in regard to the organization of its internal segments. It is apparent that the 1.9-kb HindIII sequence is only *one element* of a sequence that is surrounded by various other different repeated elements that collectively compose longer repeat *units*. Permutations of these different repeated elements could yield enormous command or recognition specificities. One might even consider them as a complex language of their own in the genome. Certain repetitive DNA elements, possibly in combination with special proteins, could thus provide ordered recognition sites on the chromosome. Two nuclear processes that can be considered in this context are chromosome replication and condensation.

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